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(54) Composition and method for the dectection of the presence of a polynucleotide sequence of interest.

(57) Described is a genetic analyses using the technique of nucleic acid hybridization. The genetic analyses includes, for example, the diagnosis of infections by foreign microbes and the detection of specific genetic traits and abnormalities. Furthermore, a technique for the detection of the presence of a polynucleotide sequence of interest is described.

COMPOSITION AND METHOD FOR THE DETECTION OF THE PRESENCE OF A POLYNUCLEOTIDE SEQUENCE OF INTEREST

The present invention relates to a wide range of genetic analyses using the technique of nucleic acid hybridization. These genetic analyses include, for example, the diagnosis of infections by foreign microbes and the detection of specific genetic traits and abnormalities. More specifically, the present invention is related to the detection of the presence of a polynucleotide sequence of interest.

A general method for the detection of a polynucleotide sequence of interest in a sample comprises:

a)   rendering at least a portion of said polynucleotide sequence of interest in single stranded form;

b)   providing a composition which comprises a first polynucleotide sequence which is substantially complementary and capable of hybridizing to said polynucleotide sequence of interest and which is labeled with a detectable marker;

c)   rendering at least a portion of said composition in substantially single stranded form;

d)   contacting said polynucleotide sequence of interest with said composition under conditions to permit hybridization; and

e)   detecting said polynucleotide sequence of
     interest by means of said detectable
     marker.

This method is often not useful when: (1) said
composition further comprises a second polynucleotide
sequence which, either in the same molecule or a
separate molecule, is not substantially complementary to
said polynucleotide sequence of interest and which is
labeled with said detectable marker; and (2) said
polynucleotide sequence of interest is potentially
contained in a sample that comprises polynucleotide
sequences not of interest. When both conditions (1) and
(2) are present, any signal detection is ambiguous as to
whether said polynucleotide sequence of interest is
detected or some polynucleotide sequences not of
interest but hybridizable to said labeled second
polynucleotide sequence are detected.

As an example, condition (1) presents itself quite
naturally when said first polynucleotide sequence is
produced by recombinant nucleic acid technology.
Recombinant nucleic acid technology allows economic
large scale production of said first polynucleotide
sequence concommitant with a second polynucleotide
sequence which is not substantially complementary to the
polynucleotide sequence of interest, the vector sequence
in this instance, on the same molecule, i.e. the
recombinant molecule. Often, it is easier or more
economical to label the entire recombinant molecule than
to label exclusively said first polynucleotide sequence.
However, this also produces a labeled second
polynucleotide sequence, i.e. the vector sequence in
this instance, which is not substantially complementary
to said polynucleotide of interest.

As another example, condition (1) presents itself when said first polynucleotide sequence is inserted, along with a second polynucleotide sequence not substantially complementary to the polynucleotide sequence of interest, into a vector to form a single recombinant molecule. This is due to the fact that it is difficult or inconvenient to separate the first polynucleotide sequence from the second polynucleotide sequence or that the boundary between said first polynucleotide sequence and said second polynucleotide sequence is not known.

Thus, in either of the two above examples, when the method for the detection of the polynucleotide sequence of interest is carried out, the labeled second polynucleotide sequence is capable of hybridizing to a complementary polynucleotide sequence that may be contained in the sample, i.e. condition (2) is present. This can generate a false positive result.

It is the object of the present invention to provide a composition comprising polynucleotide sequences and a method to use the same, which is effective in (1) detecting the presence or absence of a specific polynucleotide sequence of interest in a sample and (2) discriminating between the presence of said polynucleotide sequence of interest from the presence of polynucleotide sequences not of interest which may be contained in the sample.

The present invention provides a composition for detecting a polynucleotide sequence of interest in a sample which may contain polynucleotide sequences not of interest, which comprises:

(a) a first polynucleotide sequence wherein said first polynucleotide sequence is substantially complementary to and capable of hybridizing to said polynucleotide sequence of interest and is labeled with a first detectable marker;

(b) a second polynucleotide sequence wherein said second polynucleotide sequence is not substantially complementary to or substantially identical to said first polynucleotide sequence of interest and is labeled with said first detectable marker;

(c) a third polynucleotide sequence wherein said third polynucleotide sequence is substantially complementary to or identical to said second polynucleotide sequence and is either unlabeled or is labeled with a second detectable marker.

The present invention further provides a method, for the detection of a polynucleotide sequence of interest in the potential or actual presence of polynucleotide sequences not of interest in a sample to be examined, which comprises:

(a) providing a composition which comprises:

1. a first polynucleotide sequence wherein said first polynucleotide sequence is substantially complementary to and capable of hybridizing to said poly-nucleotide sequence of interest and

is labeled with a first detectable
marker;

2. a second polynucleotide sequence
wherein said second polynucleotide
sequence is not substantially
complementary to or substantially
identical to said first polynucleo-
tide sequence of interest and is
labeled with said first detectable
marker; and

3. a third polynucleotide sequence
wherein said third polynucleotide
sequence is substantially complemen-
tary to or identical to said second
polynucleotide sequence and is either
unlabeled or is labeled with a
second detectable marker;

(b) rendering at least a portion of, but
preferably, substantially all of said
polynucleotide sequence of interest and
said polynucleotide sequences not of
interest in said sample to be
examined in single stranded form;

(c) rendering at least a portion of, but
preferably, substantially all of said
composition in single stranded form;

(d) contacting said polynucleotide sequence of
interest and said polynucleotide sequences
not of interest in said sample to be
examined with said composition under
conditions to permit hybridization; and

(e) detecting said polynucleotide sequence of
interest by means of said first detectable
marker.

DETAILED DESCRIPTION OF THE INVENTION

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The present invention is related to the detection of a
polynucleotide sequence of interest. Preferably, the
present invention is related to the detection of a
polynucleotide sequence of interest in a diagnostic

10   sample.

The polynucleotide sequence of interest can be any
polynucleotide sequence present naturally in a sample or
added to the sample. It can be in a material in or

15   derived from a cellular system. It can be a subcellular
component as virus or viroid or virus like particule.
It can be a deoxyribonucleic acid sequence or a
ribonucleic acid sequence. It can be single stranded or
double stranded. It can be derived from a pathogen. It

20   can be a sequence of a prokaryote, such as Neisseria
meningitidis or Neisseria gonorrhoea; a eukaryote, such
as human, or a virus such as herpes simplex virus I or
herpes simplex virus II, or an extra chromosomal genetic
element such as a B-lactamase specifying plasmid. The

25   polynucleotide sequence of interest can be derived from
all or any part of the genome.

COMPOSITION OF POLYNUCLEOTIDE SEQUENCES

30   The present invention is related to compositions of
polynucleotide sequences that are useful in nucleic acid
hybridizations. The compositions comprise a first
polynucleotide sequence which is substantially
complementary to and capable of hybridizing to a

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specific polynucleotide sequence of interest and which
is labeled with a first detectable marker; a second
polynucleotide sequence that is not substantially
complementary to or substantially identical to said
polynucleotide sequence of interest and that is labeled
5   with said first detectable marker; and a third
polynucleotide sequence that is substantially
complementary to or substantially identical to said
second polynucleotide sequence and that is unlabeled or
labeled with a second detectable marker.
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The first and second polynucleotide sequences can be
present as separate molecules or can be covalently
linked. The third polynucleotide sequence is present as
a separate molecule.
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The first, second and third polynucleotide sequences of
the compositions of the present invention can be
deoxyribonucleic acid or ribonucleic acid sequences and
can be either single-stranded or double-stranded
20   molecules. The polynucleotide sequences can be produced
or obtained by any method known to those of ordinary
skill in the art, e.g., synthetic production methods or
enzymatic production methods, both _in vitro_ and _in vivo_.

25   When the method of the invention is carried out, the
presence of the third polynucleotide sequence in the
compositions of the invention serves to block the
hybridization of the second polynucleotide sequence to
any polynucleotide sequences not of interest in the
30   sample being examined, which nevertheless are
substantially complementary to said second
polynucleotide sequence. This blocking action limits
the liklihood that the second polynucleotide sequence
will generate a false positive result.
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THE SECOND POLYNUCLEOTIDE SEQUENCE AS A VECTOR
SEQUENCE

In one embodiment of the invention, the first
polynucleotide sequence, that is substantially
complementary to and capable of hybridizing to the
polynucleotide sequence of interest, is cloned into a
vector by standard recombinant nucleic acid technology
to form a recombinant molecule.

Thus, the recombinant molecule comprises the first
polynucleotide sequence and the second polynucleotide
sequence, i.e. the vector in this embodiment of the
invention.

The vector can be a plasmid, a cosmid, a bacterial virus
or an animal virus. The vector can be ribonucleic acid
or deoxyribonucleic acid. The vector can be single
stranded or double stranded.

The first polynucleotide sequence, which is part of the
recombinant molecule, can be produced economically in
large quantities inside hosts, for example, Escherichia
coli by fermentation. The recombinant molecule can be
purified by standard methods.

For detection of the polynucleotide sequence of interest
in a sample to be examined, it is desirable to label the
first polynucleotide sequence present in the recombinant
molecule with a first detectable marker. This can be
done in more than one way.

In one method, the first polynucleotide sequence is
largely separated from the vector by, for example,
cutting the recombinant molecule with a restriction
enzyme followed by agarose gel electrophoresis,

extracted and labeled. Thus, substantially only the first polynucleotide sequence and not the vector is labeled.

In a second and more economical method the entire recombinant molecule is labeled. This method can be carried out by, for example, nick translation using DNAse I and DNA Polymerase I in the presence of labeled nucleoside triphosphates. (Rigby, P. W. et. al., J. Mol. Biol. 113:237 (1977)). This results in the recombinant molecule, i.e. the first and second polynucleotide sequences, being uniformly labeled.

The second method avoids numerous drawbacks incurred by the first method. At best, the first method is extremely tedious; each step is very time consuming, especially the step of gel electrophoresis. Often the step of gel electrophoresis needs to be repeated to insure purity of the separation of the first polynucleotide sequence. Even so, the first polynucleotide sequence may still be contaminated by trace amounts of the second polynucleotide sequence, i.e. the vector sequence. In such a case, the present invention provides a benefit. Furthermore, the inherent properties of the recombinant molecules may be such that the first and second polynucleotide sequences can not be easily separated. For example, if the first polynucleotide sequence were of the same or similar size as the second polynucleotide sequence, then the separation of such two polynucleotide sequences may not be feasible.

If the method of choice for labeling the first polynucleotide sequence causes the second polynucleotide sequence to be labeled also, and if polynucleotide sequences complementary to the second polynucleotide

sequence are contained in the sample being examined, the interpretation of results of analysis based on the detection of labeled and hybridized polynucleotide sequences becomes problematic. The second polynucleotide sequence is capable of generating a false

5   positive result.

In this embodiment of the invention, wherein the entire recombinant molecule is labeled, the compositions of the invention comprise a third polynucleotide sequence. The

10   third polynucleotide sequence is either unlabeled or labeled with a second detectable marker and is substantially complementary to or substantially identical to the second polynucleotide sequence.

15   The presence of the third polynucleotide sequence in the compositions of the invention serves to block the hybridization of the second polynucleotide sequence to any polynucleotide sequences not of interest in the sample being examined, which nevertheless are

20   substantially complementary to said second polynucleotide sequence. It is believed that this blocking action is achieved in either or both of two ways.

25   First, the third polynucleotide sequence, being substantially complementary to said second polynucleotide sequence, can hybridize with the second polynucleotide sequence if said second and third polynucleotide sequences are rendered single stranded

30   and allowed to contact under conditions that permit hybridization. Second, the third polynucleotide sequence, being substantially identical to the second polynucleotide sequence, can hybridize to any polynucleotide sequences not of interest but

35   complementary to the second polynucleotide sequence and

present in the sample being examined. It is believed that either of these blocking actions inhibit the liklihood of the generation of a false positive result.

THE SECOND POLYNUCLEOTIDE SEQUENCE AS A SEQUENCE CONTIGUOUS TO BUT DISTINCT FROM THE FIRST POLYNUCLEOTIDE SEQUENCE IN THE CHROMOSOME

In another embodiment of the invention, the first polynucleotide sequence, which is substantially complementary to and capable of hybridizing to the polynucleotide sequence of interest, is covalently linked in the chromosome to the second polynucleotide sequence that is not substantially complementary or substantially identical to the polynucleotide sequence of interest, but which can potentially be substantially complementary to polynucleotide sequences not of interest in the sample being examined. The first polynucleotide sequence and the second polynucleotide sequence can have a single boundary or multiple boundaries. The boundaries can be known or unknown. In some instances, it is difficult at best and generally impossible to isolate said first polynucleotide sequence from said second polynucleotide sequence. Consequently, it is preferable to label both the first and second polynucleotide sequences.

A specific example of this embodiment of the present invention is wherein the first polynucleotide sequence is a polynucleotide sequence specific for genetic material of Neisseria gonorrhoea. A polynucleotide sequence is said to be specific for polynucleotide sequence A if and only if said polynucleotide sequence is capable of hybridizing exclusively to polynucleotide sequence A. It is known that Neisseria gonorrhoea and Neisseria meningitidis share significant nucleic acid

homology; in excess of 80% of the polynucleotide
sequence of the <u>Neisseria gonorrhoea</u> genome is
substantially complementary or substantially identical
to the polynucleotide sequence of the <u>Neisseria
meningitidis</u> genome (Kingsbury, D.T. J. Bact. (1967) 94,
5   p 870-874). In this example, a polynucleotide fragment,
derived from <u>Neisseria gonorrhoea</u> deoxyribonucleic acid,
comprising a first polynucleotide sequence specific for
<u>N</u>. <u>gonorrhoea</u> and a second polynucleotide sequence
specific for the species <u>N</u>. <u>gonorrhoea</u> and <u>N</u>.
10   <u>meningitidis</u>, is cloned into a vector to form a
recombinant DNA molecule. The first and second
polynucleotide sequences are purified from the vector
and are both labeled with a first detectable marker.
The composition of the invention provides, in addition
15   to such labeled first and second polynucleotide.
sequences, a third polynucleotide sequence which is not
labeled with said first detectable marker and which is
substantially complementary to or substantially
identical to said second polynucleotide sequence. The
20   third polynucleotide sequence, when present in suitable
amounts, will effectively prevent said labeled second
polynucleotide sequence from hybridizing to the
polynucleotide sequence not of interest, i.e. the sample
may comprise <u>N</u>. <u>meningitidis</u> DNA. Thus, a false
25   positive signal will not be generated.

The third polynucleotide sequence can be provided in one
of several ways. For example, a recombinant molecule
consisting of a vector and an inserted polynucleotide
30   sequence, isolated from <u>N</u>. <u>meningitidis</u>, which comprises
a polynucleotide sequence or sequences which are
substantially complementary or substantially identical
to said second polynucleotide sequence can be added to
the composition. Preferably, total genomic <u>N</u>.
35   <u>meningitidis</u> DNA, which comprises the third

polynucleotide sequence, can be added to the
composition.

In another specific example of this embodiment of the
present invention, the specific polynucleotide sequence
of interest is a sequence specific for herpes simplex
virus I. The first polynucleotide sequence is specific
for herpes simplex virus I DNA. The second
polynucleotide sequence which is labeled is a sequence
specific for herpes simplex virus I DNA and herpes
simplex virus II DNA. The third polynucleotide sequence
which is not labeled is a sequence substantially
complementary to or substantially identical to said
second polynucleotide sequence, i.e. that portion of
herpes simplex virus II DNA that is specific for herpes
simplex virus I DNA and herpes simplex virus II DNA, if
known. Said third polynucleotide sequence can be
provided, for example, by including in the composition,
total genomic herpes simplex virus II DNA. This
composition permits the detection of the specific
polynucleotide sequence of interest, i.e. herpes simplex
virus I DNA and inhibits the liklihood of the second
polynucleotide sequence from detecting herpes simplex
virus II DNA.

Further non-limiting examples of this embodiment of the present invention are listed in Table I below:

TABLE I

Specificity of 1st polynucleotide Sequence	Specificity of 2nd polynucleotide Sequence	Specificity of 3rd polynucleotide Sequence
Brucella abortus	Brucella abortus and B. melitenis	Brucella abortus and B. melitenis
Bordetella pertussis	B. pertussis and B. parapertussis	B. pertussis and B. parapertussis
Shigella dysenteria	Shigella dysenteria and E. coli	Shigella dysenteria and E. coli
Haemophilus influenzae	H. influenzae and H. parainfluenzae	H. influenzae and H. parainfluenzae
Mycobacterium tuberculosis	M. tuberculosis and M. bovis	M. tuberculosis and M. bovis
Pseudomonas pseudomallei	Ps. psuedomallei and Ps. mallei	Ps. psuedomallei and Ps. mallei
Salmonella typhi	S. typhi and S. typhimurium	S. typhi and S. typhimurium
Salmonella typhimurium	S. typhimurium and S. choleraesuis	S. typhimurium and S. choleraesuis

THE SECOND POLYNUCLEOTIDE SEQUENCE AS A HOST SEQUENCE

In a third embodiment of the invention, the first polynucleotide sequence, i.e. the polynucleotide

5   sequence that is substantially complementary to and capable of hybridizing to the polynucleotide sequence of interest, is produced inside hosts as an extrachromosomal polynucleotide sequence. The second polynucleotide sequence is the host polynucleotide

10   sequence.

The first polynucleotide sequence can be substantially purified by standard methods. However, it may be contaminated with a trace amount of the second

15   polynucleotide sequence, i.e. the host polynucleotide sequence. Thus, when the first polynucleotide sequence is labeled with a first detectable marker, a trace amount of the host polynucleotide sequence is also labeled. If the sample to be examined contains

20   polynucleotide sequences complementary to the second polynucleotide sequence, i.e. the host polynucleotide sequence, a false positive result can be generated. To prevent this undersirable result, the composition of the invention provides a third polynucleotide sequence

25   which, in this embodiment, is the host sequence that is not labeled with said first detectable marker.

A specific example of this embodiment of the present invention is wherein the first polynucleotide sequence

30   is an enteroinvasive plasmid, which is grown in E. coli hosts. The sample to be examined is derived from the stool of a human patient. This sample is then expected to contain E. coli polynucleotide sequence. If the labeled first polynucleotide sequence is contaminated

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even with a small amount of labeled second
polynucleotide sequence, i.e. E. coli polynucleotide
sequence, a false positive result can be generated.
However, inclusion of a third polynucleotide sequence,
i.e. unlabeled E. coli polynucleotide sequence, will
inhibit the likelihood of this undesirable result.

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Of course, in certain embodiments of the present
invention, the three embodiments discussed above,
namely, the second polynucleotide sequence as a vector
sequence, the second polynucleotide sequence as a
10   sequence chromosomally linked to the first
polynucleotide sequence and the second polynucleotide
sequence as a host polynucleotide sequence can be
combined. The third polynucleotide sequence, which is
not labeled with the first detectable marker, can
15   comprise a sequence which is substantially complementary
or substantially identical to said vector sequence and
said sequence which is chromosomally linked to said
first polynucleotide sequence, and a polynucleotide host
sequence.
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Also, in certain embodiments of the present invention
only two of the three embodiments discussed are
combined.

25   **PREFERRED MOLECULAR FORM OF THIRD POLYNUCLEOTIDE**
SEQUENCE

Experiments were performed to determine the optimal size
distribution of the third polynucleotide sequence in the
30   compositions provided by the present invention. It is
believed that the third polynucleotide sequence
fragments can be essentially any length, provided that
the fragments are long enough to form a stable hybrid.
However, a preferred embodiment of the invention is
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wherein the third polynucleotide sequence fragments are
from about 50 to about 250 nucleotides in length. These
short fragments are preferably produced by controlled
digestion with DNAse I. Alternatively, sonication or
digestion with other suitable nucleases can be used.

Experiments were also performed to determine the
appropriate amount of third polynucleotide sequence in
the composition of the present invention. It was found
that the higher the amount of third polynucleotide
sequence in the composition, the more effective said
composition was in blocking the signal generated by the
first detectable marker on the second polynucleotide
sequence. The amount of third polynucleotide sequence
to be utilized is dependent upon how the method of the
invention is carried out, as discussed hereinbelow.

DETECTABLE MARKERS AND DETECTION

A labeled polynucleotide sequence in this invention
means a polynucleotide sequence which is labeled with a
detectable marker. Any detectable markers now in use in
the art of nucleic acid hybridization or to be developed
in the future can be used. The choice of detectable
markers, is not critical to the present invention.
Suitable detectable markers include radioactive
nuclides; chemical markers including biotinated
moieties, antigens, sugars, fluors and phosphors,
enzymes, apoenzymes and cofactors, ligands, allosteric
effectors, ferritin, dyes, microspheres.

A first detectable marker is said to differ from a
second detectable marker in the context of the present
invention whenever an effective method exists and is
used, that discriminates said first detectable marker
from said second detectable marker. For example, ^{3}H and

^{32}P are both radioactive markers. They are different
detectable markers in the context of programmed
scintillation counting that discriminate higher energy
disintegrations of ^{32}P from low energy disintegration
from ^{3}H. They are not different detectable markers if
5   the scintillation counting does not discriminate the
energy of disintegration.

Another example is provided by the following pair of
labeled polynucleotide sequences: A is a polynucleotide
10   sequence labeled with biotinylated nucleotides; B is is
polynucleotide sequence labeled at the 3'-terminus with
poly T. A is detected by an avidin-horseradish
peroxidase complex which generates a color in the
presence of a suitable chromogen substrate. This method
15   is disclosed in co-pending, co-assigned U.S. Patent
Applications Serial No. 574,632, filed January 26, 1984
and Serial No. 461,469, filed January 27, 1983, the
disclosures of which are incorporated herein by
reference. B is detected indirectly through a
20   biotinylated-poly A polynucleotide bridge. The biotin
contained in said poly A polynucleotide is detected by
said avidin-horseradish peroxidase complex in the
presence of said chromogen substrate. This method is
disclosed in co-pending, co-assigned U.S. Patent
25   Application Serial No. 491,929, filed May 5, 1983. The
detection of A and B, if desired, can be separated.
If, in fact, the detection of A and B is separated, then
A and B are different detectable markers. If, in fact,
the detection of A and B is not separated, then A and B
30   are not different detectable markers.

METHODS OF USING THE POLYNUCLEOTIDE COMPOSITIONS OF THE PRESENT INVENTION

The present invention also relates to methods of using the compositions of the present invention. The
5   compositions can be used in all nucleic acid hybridization procedures. These procedures include, but are not limited to two phase hybridization and one phase hybridization. Examples of two phase hybridization are hybridization in situ and hybridization to
10 polynucleotide sequences immobilized on a transparent and nontransparent surface. An example of one phase hybridization is hybridization to polynucleotide sequences in solution. The choice of a particular procedure is not critical to the present invention.

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The genetic material of the sample to be examined is prepared as called for in the particular procedure being used, which is or will be known to a person of ordinary skill in the art. These procedures result in at least a
20 portion of the genetic material of the sample being in single stranded form, but preferably substantially all of the genetic material of the sample is in single stranded form.

25 At least a portion of the polynucleotide sequences of the compositions of the invention are rendered in single stranded form. However, it is highly preferred that said polynucleotide sequences be rendered in substantially single stranded form because
30 polynucleotide sequences in duplex form generally do not participate in hybridization. Each component, namely, the first polynucleotide sequence, the second polynucleotide sequence and the third polynucleotide sequence can be rendered in substantially single

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stranded form singly or together in any combination.
The polynucleotide sequences in said composition, thus
rendered in single stranded form, are utilized to
contact the prepared genetic material of the sample to
be examined, which has been rendered in single stranded
form, under conditions that permit hybridization. It is
highly preferred that the third polynucleotide sequence
be allowed to contact the prepared sample prior to, or
at substantially the same time as the second
polynucleotide sequence. Otherwise, given time during
which the third polynucleotide sequence is absent, the
second polynucleotide sequence can hybridize to
complementary polynucleotide sequences not of interest,
if present, in the sample being examined. This would
defeat the purpose of including the third polynucleotide
sequence in the composition and generate a false
positive result upon detection of the first detectable
marker. Within this preferred condition, there are
three preferred embodiments for practicing the method of
the invention.

In the first preferred embodiment of the method of the
invention the first, second and third polynucleotide
sequences of the composition are contacted with the
sample to be examined at about the same time. In this
embodiment it is preferred that the third polynucleotide
sequence is present in the composition in an amount by
weight from about 100 to about 1000 fold greater than
the amount of the second polynucleotide sequence in the
composition. Amounts greater than about 1000 fold
blocked essentially no more of the second polynucleotide
sequence. However, if the sample contains a greater
amount of polynucleotide sequence not of interest, but
capable of hybridizing to the second polynucleotide
sequence than the amount of second polynucleotide
sequence in the composition, then the third

polynucleotide sequence should be present in an amount
by weight from about 100 to about 1000 fold greater than
the amount of the polynucleotide sequence not of
interest but capable of hybridizing to the second
polynucleotide sequence. As a practical matter, the
latter situation is very rarely of concern.

In the second preferred embodiment of the method of the
invention, the first, second and third polynucleotide
sequences are allowed to contact each other in solution
and hybridize for a substantial amount of time so that
the hybridization of the second polynucleotide sequence
is substantially complete and that the hybridization of
the first polynucleotide sequence is not. In this
embodiment it is preferred that the third polynucleotide
sequence be present in the composition in an amount by
weight from about 100 to about 1000 fold greater than
the amount of the second polynucleotide sequence in the
composition. This excess of the third polynucleotide
sequence accelerates the hybridization of the second
polynucleotide sequence without accelerating the
renaturation of the first polynucleotide sequence. With
respect to the extra time required and the extra step
necessary to obtain a result, this embodiment is less
preferred. But this embodiment of the invention is more
preferred if the sample to be examined contains
sigificant amounts of polynucleotide sequences not of
interest but capable of hybridizing to the second
polynucleotide sequence. This is because the second
polynucleotide sequence in the composition has already
hybridized substantially to completion and can not
hybridize to any polynucleotide sequence in the sample.

In the third preferred embodiment of the method of the
invention, the third polynucleotide sequence of the
composition is allowed to contact with and hybridize

substantially to completion with the genetic material in
the sample to be examined prior to the contacting of the
second polynucleotide sequence with the genetic material
in the sample. In this embodiment it is preferred that
the third polynucleotide sequence be present in the
composition in an amount by weight from about 10 fold to
about 100 fold greater than the amount of the
polynucleotide sequence not of interest but capable of
hybridizing to the second polynucleotide sequence in the
composition. This amount is generally sufficient to
hybridize with all polynucleotide sequences not of
interest but capable of hybridizing to the second
polynucleotide sequence in the composition. This
embodiment is not preferred with respect to the extra
time required and the extra step necessary to obtain a
result. But it is preferred with respect to the
quantity of the third polynucleotide sequence required
for the composition when the sample to be examined
contains significant amounts of polynucleotide sequence
not of interest but capable of hybridizing to the second
polynucleotide sequence.

At the end of the hybridization reaction, the stable
hybrid genetic material formed is detected by means of
the first detectable marker.

In certain embodiments of this invention, after the
hybridization reaction is over, the detection step
requires a separation step which separates that part of
the composition which has hybridized to the sample being
examined from that part which has not. Such separation
can be carried out by a wash step. For example, the
sample to be examined is immobilized on a nitrocellulose
filter. Biotinylated nucleotides are used to label the
first and second polynucleotide sequences. At the end
of the hybridization reaction, the nitrocellulose filter

is washed so that unhybridized sequences in the
composition are removed. The biotinylated nucleotides
contained in the molecules which are bound to the
immobilized target are then detected by any suitable
means.

In certain embodiments of this invention, after
hybridization is over, a separation step is not
necessary in the detection process. For example, this
is the case when the detectable marker used is an
asymmetric chemiluminescent emitter/absorber system. In
this embodiment, a signal is generated only if the
labeled polynucleotide sequences in the composition have
hybridized with substantially complementary sequences in
the sample being examined. This method of detection is
disclosed in European Patent Publication 0 070 685,
published January 26, 1983. Another example utilizes a
agglutinable microsphere as the detectable marker. This
method is disclosed in co-pending, co-assigned U.S.
Patent Application Serial No. 605,022 filed April 27,
1984.

MULTIPLE DETECTION

The third polynucleotide sequence of the composition
can, if so desired, be labeled with a second detectable
marker. It is then possible to detect any third
polynucleotide sequence that hybridizes with the genetic
material in the sample, and, by inference, the presence
of a polynucleotide sequence not of interest but capable
of hybridizing to the second polynucleotide sequence of
the composition. Such detection can give a benefit if
the quantity of said polynucleotide sequence not of
interest in the sample is large, as indicated by the
signal generated by the second detectable marker, it may
become necessary to re-assess the significance of a

positive result, if any, from the first detectable
marker. This is because some fraction of the second
polynucleotide sequence may have hybridized to said
polynucleotide sequence not of interest in the sample
and contributed to the signal from the first detectable
5   marker.

The following examples are offered by way of
illustration and not by way of limitation.

10   EXAMPLE I:

Introduction

In this example of the invention, a model system was
15   used to demonstrate the detection of a polynucleotide
sequence of interest, a 9 kilobase DNA fragment of
Chlamydia trachomatis, in the presence of a
polynucleotide not of interest, pBR322.

20   Plasmids

The plasmid pCHL2 consists of a 9 kilobase BamH I
fragment from Chlamydia trachomatis cloned into the BamH
I site of the plasmid pBR322. The 9 kilobase BamH I
25   fragment has no substantial complementarity to pBR322.

Preparation of Target Samples

Sonicated pCHL2 plasmid DNA at a concentration of
30   220ug/ml in 10mM Tris-HCl pH 7.5, 0.1mM EDTA was
denatured by the addition of NaOH to a final
concentration of 0.5M. A volume of 1M Tris-HCl pH 7.5
equal to that of the alkaline DNA solution was added to
neutralize the solution. 20X SSC was then added to a
35   final concentration of 2X SSC, (1X SSC = 0.15M NaCl,

0.015M Na citrate pH 7.0). An amount equivalent to 2µg of DNA was then applied to each of 30 points on a nitrocellulose filter (previously wetted with distilled water at 65°C. and then soaked in 6X SSC) using a "minifold dot blot" apparatus. Each well was rinsed with 200 µl of 2X SSC, and the filter was then air dried and baked for 2 hours at 80°C. in vacuo. Each spot on the filter was then punched out to yield 30 small circular filters, 3/16 inch in diameter and containing 2µg bound, denatured pCHL2 DNA. As control "targets" 30 similar filters were punched from a nitrocellulose filter to which no DNA had been applied.

Preparation of Polynucleotide Composition

A. Preparation of Labeled Polynucleotide Sequences

1. Isolation of the Chlamydia Fragment

pCHL2 was digested with the restriction enzyme BamH I, and the resulting fragments separated on an 0.5% low melting temperature agarose gel. The band corresponding in size to 9.0 kb was cut from the gel, and the DNA extracted from the gel slice using sodium iodide and powdered flint glass as described by Vogelstein and Gillespie (Proc. Natl. Acad. Sci. USA 76: 615-617, 1979). An aliquot of this purified fragment was run on 0.7% agarose gel to check for contamination of the purified chlamydia fragment by the pBR322 vector. No contamination of the pure fragment was seen. However, the remaining chlamydia DNA fragment was subjected to a second round of gel electrophoresis and isolation to obviate any possibility of contamination by the pBR322 vector sequence.

2. Nick Translation of DNA Probes

To 2 µg of DNA in 10mM Tris-HCl pH 7.4, 0.1mM
EDTA, was added 10 µl of 10X nick translation
buffer (0.5M Tris-HCl pH 7.5, 0.05M $MgCl_2$,
0.1M Beta mercaptoethanol, 0.5 mg/ml bovine
serum albumin) and distilled water to a total
volume of 85 µl. To this was added 1 µl of
DNase I (freshly diluted 5000 fold in 1X nick
translation buffer from a 1 mg/ml stock
solution). The reaction was incubated at
37°C for 5 minutes and then at 68°C for 10
minutes.

1 µl each of 100mM dATP and dTTP was then added
to the reaction mixture on ice followed by
50 mCi of either ^{3}H- or ^{32}P-dCTP and dGTP.
The reaction mixture was incubated at 14°C
for 5 minutes when 2 µl of DNA polymerase I
(equivalent to 20 units) was added. After 30
minutes at 14°C the reaction was stopped by
the addition of 4 µl of 0.5M EDTA, and the
reaction placed on ice. Radioactively labeled
DNA was separated from unincorporated
nucleotides using a sephadex G50 (medium)
column.

Pure chlamydia fragment DNA was nick translated
with ^{32}P labeled nucleotides to a specific
activity of 2.1 x 10^7 cpm/µg and pBR322 was
nick translated with ^{3}H labeled nucleotides
to a specific activity of 3 x 10^6 cpm/µg.

B. **Preparation of Unlabeled Polynucleotide Sequence**

31 μg of pBR322 in 145 μl of T.E. (10mM
Tris-HCl, 0.1mM EDTA pH 7.5) was degraded to
molecules of size varying from about 25 to
about 125 base pairs using DNase I digestion.
DNase I was stored at -20°C as a 1 mg/ml
solution in 0.01N HCl, 50% glycerol and diluted
immediately before use in T.E. Digestion was
carried out in a total volume of 200 μl con-
taining: 50mM Tris pH 7.5, 1mM $MnCl_2$,
100μg/ml bovine serum albumin and 100 ng of
DNase I at 37°C for 10 minutes. The reaction
was stopped by addition of 20 μl of 0.5M EDTA
on ice. The products of digestion were
analyzed on a 4% agarose gel using HinF I
digested pBR322 as molecular weight markers.

Hybridizations with Sample DNA or Control Filters

Nitrocellulose filters with sample DNA or
control filters were pre-hybridized in
batches of 30 discs in 250 ml beakers con-
taining 50 ml of prehybridization solution at
65°C. Prehybridization was for 10 minutes
in 3X SSC, 60 minutes in 3X SSC, 5X Den-
hardt's, (5X Denhardt's = 0.1% Ficoll, 0.1%
Polyvinyl pyrolidone, 0.1% bovine serum
albumin) and 2 hours in 3X SSC, 5X Denhardt's
0.1% SDS (Na dodecyl sulfate) and 100 μg/ml
sonicated calf thymus DNA which was boiled for
5-7 minutes immediately before addition.

Hybridizations were carried out in 1.5 ml
Eppendorf tubes in a total volume of 500 μl
and contained 3X SSC, 5X Denhardt's, 0.1%
SDS and 100 μg/ml calf thymus DNA. Tubes
1 to 16 received a nitrocellulose filter

5   disc with pCHL2 DNA. Tubes 17 to 32 received
control discs with no target sequence.
Unlabeled, DNase I digested pBR322 DNA was
boiled for 5 to 7 minutes and then placed
on ice. It was added to tubes 1 to 16 and

10   17 to 32 in amounts by weight representing
0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90,
100, 150, 200, 500 and 1000 fold excess over
the amount of ^{3}H labeled pBR322 DNA. 1.25
x 10^5 cpm of boiled ^{3}H labeled DNA was

15   added to each of the hybridizations,
representing the ng of DNA contained in 53 μl.
The same number of cpm of ^{32}P labeled
chlamydia DNA was added in a volume of 6.0 μl.
Hybridizations were allowed to proceed at

20   65°C. with skaking for 16 hours.

Washing of the Filters

Each filter was washed quickly, 3 times with

25   2X SSC, 0.1% SDS at 65°C. and then washed
4 times for 30 minutes each wash at 65°C.
in 2X SSC, 0.1% SDS. Filters were then dried
under an infrared lamp, added to scintillation
vials and counted using an "Omnifluoer"

30   scintillation fluid. Vials were counted in a
Beckman LS6800 scintillation counter programmed
to discriminate ^{3}H and ^{32}P counts. One
energy spectrum channel of the scintillation
counter was set to detect low energy ^{3}H

35   disintegrations in the range of 0 to 300.

A second channel was set to detect high
energy ^{32}P disintegrations in the range of
500 to 1000. Under these conditions, spill-
over into the first channel by ^{32}P counts

5 was 1.65% that of the second channel counts
and spillover into the second channel by ^{3}H
counts was less than 0.1% that of the first
channel.

10 RESULTS

Ratio of Unlabeled DNAsed pBR322 DNA to ^{3}H Labeled pBR322 DNA	^{32}P counts	%	^{3}H counts	%
0	30,178	105	12,735	100
5	28,742	100	8,402	66
10	25,003	87	6,565	52
20	29,501	103	4,737	37
30	25,466	89	3,564	28
40	31,659	110	3,228	25
50	30,875	108	2,495	20
60	31,301	109	2,509	20
70	33,752	117	2,578	20
80	28,571	100	2,142	17
90	29,413	102	1,733	14
100	27,937	97	1,568	12
150	23,132	81	1,030	8
200	25,607	89	915	7
500	30,718	107	506	4
1000	27,580	96	267	2

Background counts from the 16 control filters ranged from
5.90 cpm to 13.60 cpm.

At a thousand fold excess unlabeled, DNAsed pBR322 DNA over ^{3}H labeled pBR322
DNA, 98% of the ^{3}H signal can be suppressed. At the same time, the results
indicate that the signal from the ^{32}P labeled Chlamydia trachomatis DNA bound
to its complementary sequence on the filter was unaffected.

EXAMPLE II:

In the example of the invention, it was demonstrated
that any amount of unlabeled pBR322 that has been
digested by DNase I to a size varying from about 25 to
5   about 125 nucleotides in length was more effective than
the same amount of unlabeled, full length linear pBR322
in blocking the hybridization of labeled pBR322 DNA to
its complementary sequence target.

Plasmids

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The same plasmids cited in Example I, namely, pCHL2,
pBR322 were used.

Preparation of Target Samples

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The 9 kilobase DNA fragment from Chlamydia trachomatis
was purified as described in Example I. Intact,
supercoiled pBR322 DNA was disrupted by brief
20   sonication. Separately, each DNA was treated
sequentially with NaOH, Tris-HCl pH 7.5 and 20X SSC as
described in Example I. 200 ng samples of pBR322 DNA or
Chlamydia trachomatis DNA were applied on nitrocellulose
filters as described in Example I. The filters were
25   then dried and baked for 2 hours at 80°C in vacuo. Each
spot on the filter was then cut out to yield 3/16 inch
diameter circular filters containing Chlamydia
trachomatis DNA or 3/16 inch x 3/16 inch square filters
containing pBR322 DNA or control filters of 3/16 inch
30   diameter containing no DNA.

Preparation of Labeled Polynucleotide Sequences

The entire plasmid pCHL2, containing the vector sequence

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pBR322 and <u>Chlamydia</u> <u>trachomatis</u> sequence, was nick
translated as previously described in Example I using
^{32}P labeled deoxynucleotides to a specific activity of
1.5 x 10^7 cpm per µg.

Preparation of Unlabeled pBR322 DNA

1. Plasmid pBR322 DNA was treated with DNase
   I in the presence of Mn++ ions as described
   in Example I to produce molecules with a
   median size of approximately 50 base pairs.

2. Plasmid pBR322 DNA was linearized by
   digestion with BamH I.

Hybridization

Filters, either discs containing chlamydia fragment DNA,
squares in containing pBR322 DNA or control discs with
no target DNA, were pre-hybridized in batches as
described previously in Example I. Hybridizations were
carried out in a volume of 500 ul in 1.5ml Eppendorf
tubes as in Example I. 8.8ng of nick translated pCHL2
DNA (1.25 x 10^5 cpm) was added to each hybridization.
The first 26 hybridizations each contained one circular
and one square filter. In addition, unlabeled, DNased
pBR322 DNA or BamH I digested pBR322 DNA was added in
varying amounts and corresponding to a 0, 5, 10, 20, 30,
40, 50, 60, 70, 80, 90, 100, 150, 200, 500 or 1000 fold
excess by weight over the labeled probe. 26 control
hybridizations were set up in the same way except that
one nitrocellulose filter which contained no target DNA
sequence was added to each hybridization. Hybridization
and washing conditions were as previously described in
Example I.

Filters were dried and each filter counted separately in a scintillation counter.

RESULTS

Gravimetric ratio of un-labeled pBR322 DNA/labeled pCHL2 DNA	Using Unlabeled, DNAsed pBR322 DNA		Using BamH I digested full length, unlabeled pBR322 DNA	
	cpm Bound to Chlamydia target	cpm Bound to pBR322 target	cpm Bound to Chlamydia target	cpm Bound to pBR322 DNA
0	13,211	6,696	14,471	6,824
5	12,983	4,318	13,066	5,075
10	15,442	3,602	12,679	4,328
20	12,441	2,594	12,942	2,997
30	12,206	2,066	-	-
40	10,630	1,602	-	-
50	13,940	1,356	12,212	1,747
60	13,392	1,451	-	-
70	14,877	1,234	-	-
80	12,635	1,197	-	-
90	13,108	1,115	-	-
100	14,734	1,016	12,850	1,953
150	14,247	764	10,846	2,072
200	15,659	649	11,909	1,849
500	12,509	324	12,078	1,577
1000	13,261	200	10,620	1,056

EXAMPLE III:

This example illustrates how a recombinant plasmid, consisting of a DNA fragment from Neisseria gonorrhea cloned into the vector pBR322, can be used to detect N. gonorrhea DNA even if said fragment comprises a sequence that is a substantially complementary to some sequence of Neisseria meningitidis.

Plasmids

pAL1 consists of a 1.1kb fragment of N. gonorrhea DNA
cloned into the Pst I site of pBR322 by the homopolymer
dG:dC tailing method.

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Preparation of Target Samples

Chromosomal DNA from N. gonorrhea or N. meningitidis was
prepared by the method of Marmur (J. Mol. Biol. 3:

10   208-218, (1961). $2\mu g$ of N. gonorrhea DNA or $2\mu g$ of N.
meningitidis DNA were immobilized on each of 16 circular
and square nitrocellulose filters respectively as
described in Examples I and II. Control filters contain
no DNA.

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Preparation of labeled Polynucleotide Sequence

Plasmid pAL1 DNA was labeled by nick translation as
described previously in Example I using four ^{32}P labeled

20   nucleotides. The specific activity of the labeled DNA
was 2.7×10^8 cpm/μg. 1.25×10^5 cpm of the
radioactively labeled probe were to be added to each
500ul hybridization reaction, corresponding to 0.47μg of
probe DNA.

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Preparation of Unlabeled Polynucleotide Sequences

1. Since the radioactively labeled
polynucleotide sequences pAL1 used in this
experiment contained the vector plasmid

30   pBR322, unlabeled pBR322, was used in a
1000 fold excess by weight in the
hybridization reactions to block any
unwanted signal from this component of the

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labeled polynucleotide sequence. The
unlabeled pBR322 DNA was prepared by
sonication of plasmid DNA to produce linear
fragments of a median size of approximately
300 base pairs.

2. Chromosomal DNA from a strain of N.
   meningitidis, which had been shown to
   cross-react with the probe pAL1 was
   disrupted by sonication to produce linear
   molecules of a median size of approximately
   300 base pairs. DNA was ethanol
   precipitated and resuspended at a
   concentration of 10mg/ml in distilled
   water.

Hybridization

Hybridizations were set up at 65°C and contained 3X SSC,
5X Denhardt's, 0.1% SDS and 100ug/ml calf thymus DNA.
Tubes 1 to 16 received one circular filter with 2μg of
N. gonorrhoeae DNA and one square filter with 2μg of N.
meningitidis DNA. Tubes 17 and 32 received one control
filter. Unlabeled pBR322 DNA was added at 1000 fold
excess and unlabeled N. meningitidis DNA was added at a
0, 125, 250, 500, 103, 2×10^3, 3.9×10^3, 7.8×10^3,
1.6×10^4, 6.25×10^4, 1.25×10^5, 2.5×10^5, 5×10^5,
10^6 and 2×10^6 fold excess over the amount of pAL1
probe DNA. DNA was boiled for 5 to 7 minutes and then
placed on ice before addition to hybridization
reactions. Hybridization was carried out for 16 hours
at 65°C.

The filters were then rinsed 3 times with 2 X SSC, 0.1%
SDS at 65°C. Filters were dried under an infrared lamp
and counted separately in a Beckman LS6800 scintillation
counter using a standard scintillation cocktail.

RESULTS

Relative Conc. Suppressor/probe	cpm bound to N. gonnhorea filter	%	cpm bound to N. meningitidis filter	% of Counts bound to n.g. filter at 0 suppression
0	3,084	100	291	9.4
125	3,241	105	253	8.2
250	3,072	100	250	8.1
500	3,512	114	265	8.6
10^3	3,429	111	228	7.4
2×10^3	2,489	81	195	6.3
3.9×10^3	2,467	80	165	5.4
7.8×10^3	2,727	88	136	4.4
1.6×10^4	2,786	90	95	3.1
3.13×10^4	2,295	74	67	2.2
6.25×10^4	2,385	77	42	1.4
1.25×10^5	2,238	73	64	2.1
2.5×10^5	2,106	68	43	1.4
5×10^5	1,195	39	26	0.8
10^6	821	27	14	0.5
2×10^6	741	24	3	0.1

A number of important conclusions may be drawn from these results:

1. The number of counts bound to the filter containing N. meningitidis DNA was 9.4% of the counts bound to the filter containing N. gonorrhoeae DNA. Therefore, some portion of the 1.1kb fragment of N. gonorrhoeae DNA contained in pAL1 was substantially complementary to N. meningitidis target.

2. The addition of increasing amounts of un-
   labeled N. meningitidis DNA in
   hybridization reactions suppressed hybrid-
   ization of labeled pAL1 to the N.
   gonorrhoeae target as well as the N.
   meningitidis target. When unlabeled
   N. meningitidis DNA was added to a
   2×10^6 fold excess, the hybridization of
   the pAL1 probe to the N. gonorrhoeae
   target DNA is reduced to 24% of the normal
   value. Under the same conditions, the
   hybridization of the pAL1 probe to N.
   meningitidis target DNA is reduced to
   almost undetectable levels.

3. An amount of unlabeled N. meningitidis
   DNA can be chosen (3 x 10^4 fold excess,)
   at which cross-reactivity of the probe
   with N. meningitidis chromosomal DNA is
   reduced to only 2% while the probe retains
   74% of its affinity for N. gonorrhea
   chromosomal DNA.

4. This example illustrated how this invention
   can be used to avoid mis-identifying N.
   meningitidis DNA for N. gonorrhea
   DNA.

It will be appreciated that the operability of the
invention does not depend on the use of the specific
microorganisms mentioned in the samples.
These microorganisms merely serve as examples how to
carry out the invention.

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Claims

      1.   A composition for detecting a poly-
nucleotide sequence of interest in a sample which
may contain polynucleotide sequences not of interest
which comprises:

          (a) a first polynucleotide sequence
wherein said first polynucleotide sequence is
substantially complementary to and capable of
hybridizing to said polynucleotide sequence of
interest and is labeled with a first detectable
marker;

          (b) a second polynucleotide sequence
wherein said second polynucleotide sequence is
not substantially complementary to or substan-
tially identical to said polynucleotide sequence
of interest and is labeled with said first
detectable marker; and

          (c) a third polynucleotide sequence
wherein said third polynucleotide sequence is
substantially complementary to or identical to
said second polynucleotide sequence and is
either unlabeled or labeled with a second
detectable marker.

      2.   The composition according to Claim 1,
wherein said first polynucleotide sequence and said
second polynucleotide sequence are derived from a
recombinant molecule wherein said second polynucleo-
tide sequence comprises a vector polynucleotide
sequence.

      3.   The composition according to Claim 1,
wherein said first polynucleotide sequence is
covalently linked to said second polynucleotide
sequence in a chromosome.

4. The composition according to Claim 3 wherein said first polynucleotide sequence is specific for a polynucleotide sequence selected from

<u>N. gonorrhoea</u>, herpes simplex virus I, herpes simplex virus II, <u>Brucella abortus</u>, <u>Bordetella pertussis</u>, <u>Shigella dysenteria</u>, <u>Haemophilus influenzae</u>, <u>Mycobacterium tuberculosis</u>, <u>Pseudomonas pseudomallei</u>, <u>Salmonella typhi</u>, <u>Salmonella typhimurium</u> or <u>N. meningitidis</u>.

5. The composition according to Claim 1, wherein said second polynucleotide sequence is a host polynucleotide sequence.

6. The composition according Claim 1, wherein said third polynucleotide sequence is unlabeled.

7. A method for the detection of a poly-nucleotide sequence of interest in the potential or actual presence of polynucleotide sequences not of interest which comprises:

   (A) providing a composition which comprises:

      (i) a first polynucleotide sequence wherein said first polynucleotide sequence is substantially com-plementary to and capable of hybridizing to said polynucleotide sequence of interest and is labeled with a first detectable marker;

     (ii) a second polynucleotide sequence wherein said second polynucleotide sequence is not substantially complementary to or substantially identical to said polynucleotide sequence of interest and is

labeled with said first detectable
marker;

(iii) a third polynucleotide sequence
wherein said third polynucleotide
sequence is substantially comple-
mentary to or identical to said
second polynucleotide sequence
and is either unlabeled or labeled
with a second detectable marker;

(B) rendering at least a portion of said
polynucleotide sequence of interest
and said polynucleotide sequences not
of interest in single stranded form;

(C) rendering at least a portion of said
composition in single stranded form;

(D) contacting said polynucleotide sequence
of interest and said polynucleotide
sequences not of interest in said
sample with said composition under
conditions to permit hybridization;
and

(E) detecting said polynucleotide sequence
of interest by means of said first
detectable marker.

8.   The method according to Claim 7,
wherein substantially all of said polynucleotide
sequence of interest and said polynucleotide
sequences not of interest are rendered in single
stranded form and substantially all of said com-
position is rendered in single stranded form.

9.   The method according to Claim 8,
wherein said polynucleotide sequence of interest and
said polynucleotide sequences not of interest in
said sample are contacted with said third polynucleo-
tide sequence prior to contacting polynucleotide

sequence of interest and said polynucleotide
sequences not of interest in said sample with said
first polynucleotide sequence and said second
polynucleotide sequence.

    10.   The method according to Claim 8, which
further comprises contacting said second polynucleo-
tide sequence with said third polynucleotide sequence
under conditions to permit hybridization after said
composition is rendered into substantially single
stranded form, but before said polynucleotide sequence
of interest and said polynucleotide sequences not of
interest are contacted with said composition.